Biochemical and Biophysical Research Communications 371 (2008) 168–171

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Single-amino-acid mutation in the HA alters the recognition of H9N2 influenza virus by a monoclonal antibody

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ARTICLE INFO

*Article history:*
Received 4 April 2008
Available online 18 April 2008

*Keywords:*
Avian influenza virus
H9N2 subtype
Genetic basis
Antigenic variation

ABSTRACT

We explored the molecular basis of antigenic variation by comparing two H9N2 subtype avian influenza viruses, A/Chicken/Shandong/6/96 (CK/SD/6) and A/Chicken/Guangxi/10/99 (CK/GX/10), that react differently to a monoclonal antibody C/B3. To assess the genetic basis for this antigenic difference, we used reverse genetics to generate a series of chimera and mutants of these two viruses. We found that a single-amino-acid substitution of asparagine for serine at position 145 (S145N) in the HA protein prevents the reaction of CK/SD/6 virus with C/B3. Substitution of serine for asparagine at the same position (N145S) enables the CK/GX/10 to react with C/B3 in hemaglutinin inhibition, immunofluorescence and neutralization assays. We further demonstrated that the amino acid N145 in the H9 HA protein is glycosylated. Our results provide experimental evidence that the glycosylation of HA oligosaccharide attachment sites implicated in antibody binding could have a role in antigenic variation.



Influenza virus has two surface glycoproteins, hemagglutinin (HA), and neuraminidase (NA). The HA glycoprotein of influenza virus is the main target of host neutralizing antibodies [1]. Amino acid sequence variation resulting in altered antigenic properties of the HA accompany recurrent epidemics of H1 and H3 subtype influenza in humans [2–5]. Previous studies have determined that amino acids located in or near antigenic sites of the HA trimer had greater variability, a higher ratio of sense mutation, and a greater correlation with the antigenic drift of H1 and H3 human influenza viruses [2,6–8]. However, knowledge regarding the genetic determinants of antigenic drift for other influenza virus subtypes remains very limited.

H9N2 subtype avian influenza virus (AIV) has widely circulated in the world since first being detected in turkeys from Wisconsin in 1966 [9]. In China, H9N2 AIV was first isolated from chickens in Guangdong province in 1994 [10]. The virus then spread to several other southern provinces and resulted in severe economic losses for the poultry industry. We previously reported a detailed analysis of the H9N2 viruses isolated from domestic poultry in China and found that these viruses exhibited extensive antigenic diversity [11]. In the present study, we investigated two H9N2 AIVs, A/chicken/Shandong/6/96 (CK/SD/96) and A/chicken/Guangxi/10/99 (CK/GX/99), that were isolated from China in 1996 and 1999, respectively. These two viruses react differently to an H9N2 monoclonal antibody (MAb), C/B3. The CK/SD/96 virus reacts to C/B3 in the hemagglutinin inhibition (HI) test, microneutralization test, and immunofluorescence assay, but CK/GX/99 does not react with C/B3 in these same tests. We used reverse genetics to determine the molecular basis for the difference in the reaction of these two viruses to C/B3. We determined that a specific amino acid in the HA protein at position 145 confers an antigenic difference to these avian H9N2 viruses.

## Materials and methods

*Viruses and cells*. The H9N2 viruses, CK/SD/96 and CK/GX/99, used in this study were isolated from domestic poultry and have been described previously [11]. Madin–Darby canine kidney (MDCK) cells and human embryonic kidney cells (293T) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and were incubated at 37 °C in 5% $CO_2$.

*Construction of plasmids*. We used a 12 plasmid reverse genetics system for virus rescue [12]. We inserted the HA and NA cDNAs of CK/SD/96 and CK/GX/99 viruses into the mRNA-viral RNA (vRNA) pBD vector [13] and constructed bidirectional expression plasmids. The chimeric HA genes were generated by cutting and switching the BglII, NsiI, and HindIII fragments of the plasmids pBD-CK/SD/96HA and pBD-CK/GX/99 (Fig. 1). Mutations were introduced into the HA gene by RT-PCR using a set of primers (sequences available upon request). The six internal vRNA genome fragments and four mRNA expression plasmids of PB2, PB1, PA, and NP of the A/Puerto Rico/8/34 (PR8) virus were kindly provided by Drs. Brownlee and Fodor from Oxford University.

*Virus rescue*. Virus rescue was performed as previously described [13]. Briefly, DNA and transfection reagent were mixed (2 μl of Lipofectamine 2000 per μg of DNA), incubated at room temperature for 30 min, and added to an 80% confluent monolayer of cells in six-well plates. Sixteen hours later, the DNA-transfection reagent mixture was replaced by Opti-MEM (GIBCO/BRL, Carlsbad, CA) containing

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doi:10.1016/j.bbrc.2008.04.045

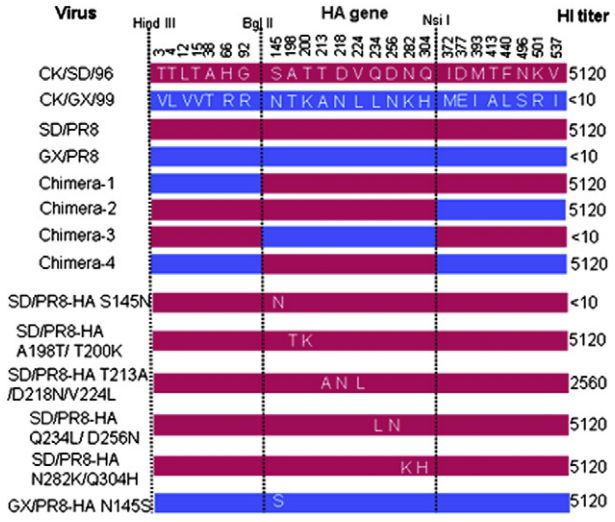


**Fig. 1.** Rescued H9N2 mutant viruses and their reaction with MAb C/B3 by the hemagglutinin inhibition test. The color of the bar indicates the origin of the HA gene as follows: red, CK/SD/96; blue, CK/GX/99. The CK/SD/96 and CK/GX/99 viruses have a 25 amino acid difference in their HA protein, and the corresponding amino acids are shown as single-letter abbreviations with their positions numbered above. The mutated amino acids are shown in bold and italics.

TPCK-Trypsin (2 μg/ml). Forty-eight hours after the transfection, the supernatants were harvested and inoculated into 10-day-old SPF embryonated chicken eggs for virus propagation. Viruses were detected by the hemagglutinin assay and the HA and NA genes were fully sequenced to ensure the absence of unwanted mutations.

*Hemagglutinin-inhibition test*. HI tests were done by standard methods [14], using anti-HA monoclonal antibody C/B3 and 0.5% chicken erythrocytes.

*Micro-neutralization assay*. Aliquots of 2-fold serially diluted C/B3 MAb were incubated with 100$TCID_{50}$ of the tested virus at 4 °C for 2 h and then added to monolayer of MDCK cells in 96-well plates. After incubation for 48 h at 37 °C, the cells were examined for cytopathic effects (CPE) and the antibody titer was defined as the reciprocal of the highest serum dilution that completely inhibited CPE.

*Immunofluorescence analysis*. About 70–80% confluent MDCK cells in 12-well cell culture plates were infected with tested virus at a MOI of 0.2. At 48 h post-infection, the infected cells were fixed with 1:1 cold methanol–acetone (vol/vol) for 20 min at −20 °C. The cells were rinsed three times in PBS and blocked for 30 min in PBS containing 1% BSA serum (Sigma). The methanol–acetone-fixed cells were incubated with MAb C/B3, with anti-H9 sera, or with the noninfected (NI) SPF chicken sera for 1 h at 37 °C, rinsed three times with PBS and incubated for 1 h at 37 °C with fluorescein-labeled goat anti-mouse IgG (Southern Biotech) or fluorescein isothiocyanate-conjugated rabbit anti-chicken IgG (Sigma). The cells were then rinsed three times with PBS and once with deionized water and 0.5 ml of 90% glycerol buffer was added to the cells. Cells were analyzed with a Leica DMIRE2 fluorescence microscope (Leica).

*Western blot*. Viruses were propagated in the allantoic cavity of embryonated SPF chicken eggs and purified from the allantoic fluid by centrifugation. The concentrated virus was resuspended with PBS. The samples were analyzed by SDS–PAGE and Western immunoblotting as described previously [15]. The chicken antisera induced by the H9N2 AIV was used as a primary antibody, and horseradish peroxidase-conjugated rabbit anti-chicken immunoglobulin G (IgG) (Sigma, St. Louis, MO) was used as a second antibody.

## Results and discussion

### *Reaction of the H9N2 viruses with MAb C/B3 in HI, neutralization and immunofluorescence assays*

We previously generated a MAb, C/B3, from an early H9N2 avian influenza virus isolate CK/SD/96. We find that C/B3 displays a high inhibition titer against CK/SD/96 in the HI and neutralization tests (Figs. 1 and 2), and it binds to CK/SD/96 virus in the immunofluorescence assay (Figs. 1 and 2). However, the antibody C/B3 does not react at all with the CK/GX/99 virus in these assays (Figs. 1 and 2).

### *Rescued reassortant viruses retained the antigenic property of wild-type viruses*

We inserted HA and NA full-length cDNA of CK/SD/96 and CK/GX/99 into the viral RNA-mRNA bidirectional expression plasmid vector pBD as described in Materials and methods. We transfected 293T cells with HA and NA bi-directional transcription plasmids of CK/SD/96 or CK/GX/99, together with the six internal gene transcription plasmids and the four protein expression plasmids of PB2, PB1, PA, and NP of A/PR/8/34 (PR8) as described previously [12]. Two recombinant viruses derived from CK/SD/96 and CK/GX/99 were rescued and were designated SD/PR8 and GX/PR8, respectively. After confirmation by sequence analysis, we tested their antigenic property by HI test, neutralization test and immunofluorescence assay (Figs. 1 and 2). The results show that the rescued recombinant virus SD/PR8 reacted well with the C/B3 MAb in all assays, but GX10/PR8 did not react with C/B3 at all in these assays. These results indicated that the rescued viruses maintained the antigenic property of the wild-type viruses.

### *Amino acid at position 145 of HA alters the reaction of H9N2 viruses to the MAb C/B3*

There is a 25 amino acids difference in the HA proteins of CK/SD/96 and CK/GX/99 (Fig. 1). To investigate the molecular basis that contributes to the antigenic difference of CK/SD/96 and CK/GX/99 viruses, we first generated three chimeric viruses that contained different HA fragments in the SD/PR8 background and confirmed their reaction to the MAb C/B3 by HI tests. The chimera-1 virus (SD/PR8 virus possessing the portion of the CK/GX/99 HA gene encoding the amino terminus of the protein) and chimera-2 (SD/PR8 virus possessing the portion of the CK/GX/99 HA gene encoding the carboxy terminus of the protein) reacted well to C/B3, but the replacement of the middle portion of HA gene with that of CK/GX/99 virus (chimera-3) resulted in the loss of recognition by the C/B3 antibody (Fig. 1). We next generated the chimera-4 virus (GX/PR8 virus possessing the middle portion of the CK/SD/96 HA gene) and found that it regained the reactivity to the MAb C/B3 (Fig. 1). These results indicated that it is the middle portion of the HA gene that contributes to the reaction of the CK/SD/96 to MAb C/B3.

The CK/SD/96 and CK/GX/99 differed at 10 amino acids in the middle portion of the HA protein (Fig. 1). To pinpoint which amino acid(s) contributed to the differential reaction of CK/SD/96 and CK/GX/99 viruses to C/B3, we generated five viruses that contained single, double or triple mutations in the HA protein in the background of SD/PR8 virus and investigated their reactions to C/B3 virus by HI test. As shown in Fig. 1, only the virus that contained the single mutation of S to N at position 145 (SD/PR8-HA S145N) lost the ability to react with C/B3. All other mutants (SD/PR8-HA A198T/T200K, SD/PR8-HA T213A/D218N/V224L, SD/PR8-HA Q234L/D256N, and SD/PR8-HA N282K/Q304H) react with C/B3 at the same level as the wild-type CK/SD/96 and reassortant SD/PR8 viruses do. We then generated another mutant in the GX/PR8 background that contained a single mutation of N to S at position 145 (GX/PR8-HA N145S) and found that this virus could be inhibited by C/B3 in the HI test (Fig. 1). These results demonstrated that the amino acid at position 145 of HA alters the reaction of H9N2 virus to the MAb C/B3. This result was further confirmed by neutralization test and immunofluorescence assay (Fig. 2).

### *Amino acid at position 145 affects the mobility of the HA protein of H9N2 viruses*

The HA of influenza virus is a glycoprotein that has several potential glycosylation sites (PGS), but not all of these potential sites

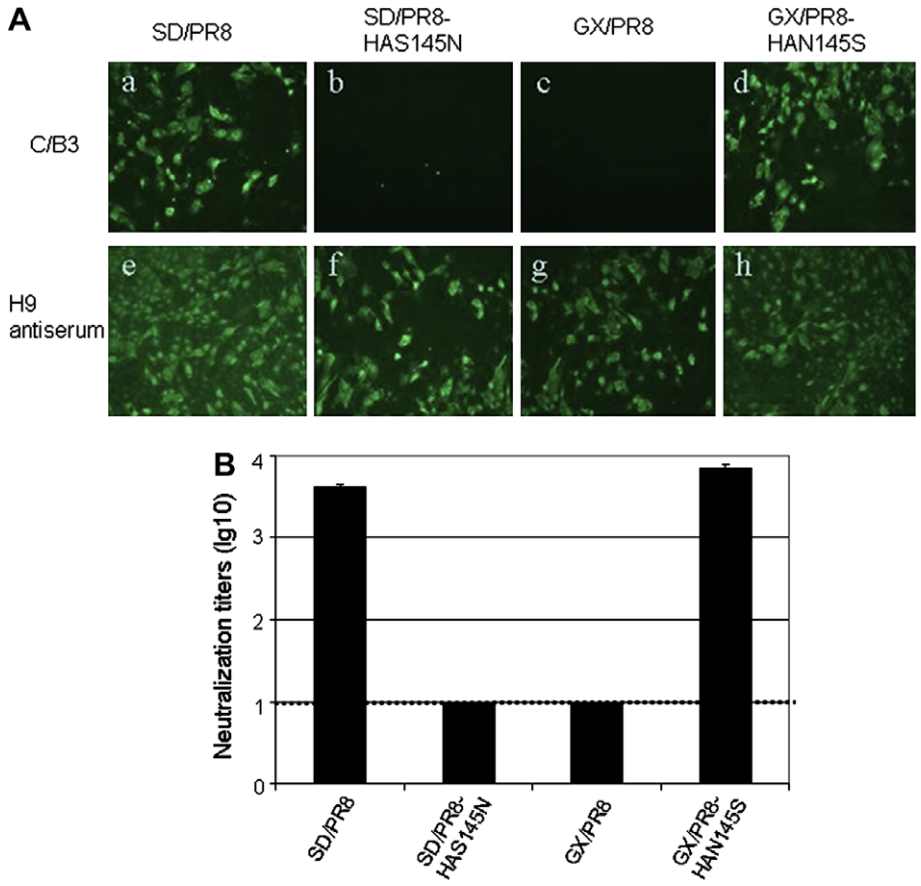


**Fig. 2.** Reaction of H9N2 avian influenza viruses with the C/B3 by immunofluorescence analysis and neutralization test. (A) Immunofluorescence analysis. Confluent MDCK cells were infected with the tested viruses indicated at a MOI of 0.2. The infected cells were fixed and probed with MAb C/B3 (a, b, c, d) or with chicken anti-H9 AIV antisera (e, f, g, h) followed by incubation with fluorescein isothiocyanate-conjugated fluorescein-labeled goat anti-mouse IgG (Southern Biotech) (a, b, c, d) or rabbit anti-chicken IgG (Sigma) (e, f, g, h). Cells were analyzed with a Leica DMIRE2 fluorescence microscope (Leica). (B) Neutralization test. The dashed line indicates the limit of detection.

are glycosylated. The amino acid Asn at the position 145 of the HA protein of CK/GX/99 virus forms a potential glycosylation site—NGT—at amino acids 145–147, while there is no such potential glycosylation site at the corresponding amino acid positions in the HA protein of the CK/SD/96 virus. To investigate whether the PGS at position 145–147 of the HA protein of CK/GX/99 is glycosylated, we performed Western analysis of the HA polypeptide of the four viruses, SD/PR8, SD/PR8-HA S145N, GX/PR8, and GX/PR8-HA N145S. As shown in Fig. 3, the SD/PR8-HA S145N shows a decrease in mobility of the HA1 polypeptide as a result of a single amino acid mutation of S145N. In contrast, the GX/PR8-HA N145S shows an increase in mobility of the HA1 polypeptide as a result of a single amino acid mutation of N145S (Fig. 3). The altered mobility suggests that the PGS at position 145–147 in the HA proteins of CK/GX/99 and SD/PR8-HA S145N virus may be glycosylated.

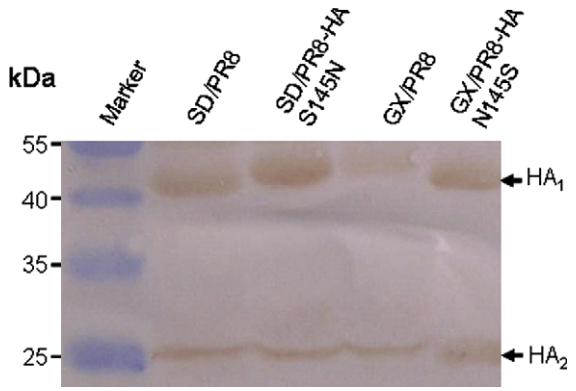


**Fig. 3.** Western blot analyses of H9N2 avian influenza HA protein. Lysates of the purified H9N2 viruses were incubated with chicken anti-H9N2 antiserum. Binding was visualized with 3,3′-diaminobenzidine reagent after incubation with peroxidase-conjugated secondary antibodies. Locations of marker proteins are indicated on the left, and the HA1 and HA2 of AIV hemagglutinin are indicated on the right.

We further tested the reactions of 18 H9N2 viruses with the MAb C/B3, and found that two other viruses, A/Guangxi/9/99 and A/Chicken/Hebei/Shijiazhang/31/00, containing a PGS at position 145–147 of HA did not react with C/B3. All other viruses that lacked a PGS at position 145–147 reacted well to the antibody with the exception of A/chicken/Shijiazhuang/2/98 virus whose reaction titer to C/B3 was only 80 (Table 1). These data suggested that the oligosaccharide side chain attached to the amino acid positions 145–147 prevents the reaction of the virus with MAb C/B3.

Among the 16 antigenic subtypes of influenza virus, only a few of the HA subtypes have been structurally characterized and antigenically mapped [16–18]. The HA molecule of the H3 subtype was first characterized by sequencing the HA of antigenically drifted variants and escape mutants, and the antigenic epitopes were mapped on the molecule's three-dimensional structure [19]. According to the antigenic structure of H3 HA, the antigenic site A in the H9 HA is centered around a protruding loop containing residues 141, 145, and 148–155 [20,21]. Antigenic site A of CK/GX/99 contains the PGS located at amino acid position 145 and it is possible that glycosylation of this site interferes with the ability of MAb C/B3 to recognize the HA protein and suggests that the epitope recognized by MAb C/B3 is located in the antigenic site A. This is further supported by the reduced affinity of MAb C/B3

**Table 1**
Cross reaction of H9N2 viruses with MAb C/B3 by hemagglutinin inhibition (HI) test

| Virus[a] | HI titers[b] | PGS at 145 | Amino acids in antigenic region A[c] |
|---|---|---|---|
| | | | 141,145,148–155 |
| CK/SD/6/96 | 5120 | no | N, S, SKACSDSF |
| CK/GX/10/99 | < | yes | -, N, - - - - - - - - |
| CK/SD/7/96 | 5120 | no | -, -, - - - - - - - - |
| DK/NJ/2/97 | 5120 | no | -, -, - - - - - - - - |
| CK/GD/5/97 | 5120 | no | -, -, - - - - - - - - |
| CK/GD /6/97 | 5120 | no | -, -, - - - - - - - - |
| CK/SJZ/2/98 | 80 | no | -, -, - R - - - - - - |
| CK/BJ/8/98 | 5120 | no | -, -, - - - - - - - - |
| CK/HN/5/98 | 5120 | no | -, -, - - - - - - - - |
| CK/GX/10/99 | < | yes | -, N, - - - - - - - - |
| CK/NX/4/99 | 5120 | no | -, -, - - - - - - - - |
| CK/GD/4/00 | 5120 | no | -, -, - - - - - - - - |
| CK/HB/31/00 | < | yes | -, N, - - - - - - - - |
| CK/SH/10/01 | 5120 | no | -, -, - - - - - - - - |
| CK/JL/53/01 | 5120 | no | -, -, - - - - - - - - |
| CK/GD/47/01 | 5120 | no | -, -, - - - - - - - - |
| CK/HLJ/48/01 | 5120 | no | -, -, - - - - - - - - |
| CK/GD/56/01 | 5120 | no | -, -, - - - - - - - - |
| CK/HN/43/02 | 5120 | no | -, -, - - - - - - - - |
| CK/GD/21/02 | 5120 | no | -, -, - - - - - - - - |

[a] Virus abbreviations—animals: chicken, CK; duck, DK. Place: Guangdong, GD; Shandong, SD; Nanjing, NJ; Beijing, BJ; Henan, HN; Shijiazhuang, SJZ; Guangxi, GX; Ningxia, NX; Hebei, HB; Shanghai, SH; Jilin, JL.
[b] Monoclonal antibody was diluted 10-fold. <, Below the level of detection of 10.
[c] Amino acids are shown by single letter abbreviations. -, the amino acid is the same as is found in the CK/SD/6/96 virus.

to the CK/SJZ/2/98 virus, which has a K149R mutation in the protruding loop (Table 1) and may influence the structure of the epitope.

A previous study used escape mutants to partially map the antigenic structure of H9 HA protein [18]. It revealed that there are two partially overlapping antigenic sites at the top of H9 HA molecule, and that the residue 127 contributes to the overlap of the epitopes. Amino acid 145 in our study was counted as 127 (H9 HA1 number, without signal peptite) in Kaverin's report. In our present study, we only tested the effect of a mutation S145N within the HA protein on the ability of MAb C/B3 to recognize the virus, and it is remains to be investigated if this mutation affects the reaction between the virus with other MAbs recognizing epitopes in other antigenic locations.

In summary, we used reverse genetics to explore the genetic basis for the different reaction of two H9N2 avian influenza viruses to the monoclonal antibody C/B3. Evidence is presented that is consistent with the possibility that an oligosaccharide side chain attached at position 145 of the HA polypeptide chains of the CK/GX/96 virus is directly responsible for the reduced affinity between the virus and the MAb C/B3 produced against the vaccine strain CK/SD/96. Our results are consistent with the hypothesis put forth by others that the glycosylation of oligosaccharide attachment sites in the HA protein is implicated in antibody binding and could have a role in antigenic variation.

## Acknowledgements

We thank Gloria Kelly for editing the manuscript, Nancy Cox and Kanta Subbarao for providing the plasmid pBD, George Brownlee and Ervin Fodor for providing the PR8 plasmids. This work was supported by Chinese National S&T Plan Grant 2004BA519A-57, the Chinese National Key Basic Research Program (973) 2005CB523005 and 2005CB523200.

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